EXHIBIT D

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Shone, *et al.*
Title:	Recombinant toxin fragments
Appl. No.:	10/527411
Filing Date:	11/10/2005
Examiner:	Archie, Nina
Art Unit:	1645
Confirmation Number	7312

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

DECLARATION UNDER 37 CFR § 1.132

Sir:

I, Clifford Charles Shone, do hereby declare and state as follows:

I am currently employed at Health Protection Agency, Porton Down, Salisbury, Wiltshire, UK, SP4 0JG, as a Scientific & Programme Leader. I am also an inventor on the present application (CA 2,264,191).

I have been actively undertaking research in the technical field of clostridial neurotoxins for over 10 years. As Scientific & Programme Leader at Health Protection Agency, I am responsible for several projects concerned with the exploitation of protein toxins including vaccine development and therapeutic antibodies. A copy of my c.v. is attached showing further details of my academic and industrial experience.

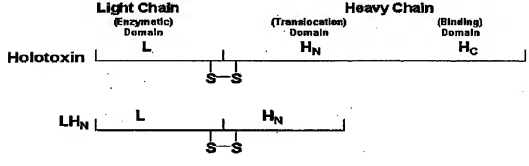
I am familiar with the prosecution history of US 10/527411 and have read the Office Action dated 3 August 2009.

In this Declaration, I confirm that it would have been routine for a skilled person to identify "fragments" and "variants" of the recited single chain polypeptide that possess the desired technical functions.

Overview of Single Chain Polypeptide:

The single chain polypeptides recited in Claim 1 of the application are defined by reference to specific amino acid sequence SEQ ID NOs.

These SEQ ID NOs comprise a clostridial neurotoxin light chain polypeptide sequence and a clostridial neurotoxin heavy chain H_N polypeptide sequence. However, they lack a functional clostridial neurotoxin heavy chain H_C polypeptide (and may lack the H_C polypeptide altogether). Accordingly, these SEQ ID NOs are termed "LH_N polypeptides", and are illustrated in the diagram below (in comparison to botulinum neurotoxin holotoxin, which also includes a functional H_C polypeptide).



The structure of clostridial neurotoxins was well known prior to the present invention.

I refer, for example, to the accompanying publication by Kurazono *et al.* (1992), which describes the minimum Domains required for cleavage activity (eg. proteolytic enzyme activity) of a clostridial neurotoxin L-chain.

By way of exemplification, Table II on page 14726 of Kurazono *et al.* illustrates a number of L-chain deletion mutants (both amino-terminal and carboxy-terminal L-chain deletion mutants are illustrated).

Kurazono *et al.* also discusses the structure of clostridial neurotoxin heavy chains, together with the functions associated with the H_N domain of the heavy chain (see, for example, the bottom illustration in Fig. 1 on page 14722 of Kurazono *et al.*).

Fragments of the single chain polypeptide

The "fragments" are defined in part (II) of Claim 1 by reference to being at least 80% the length of the amino acid sequence of the single chain polypeptide provided in part (I).

Claim 1 further qualifies the single chain polypeptide fragment as having the native protease activity of clostridial neurotoxin light chain. Thus, Claim 1 does not embrace <u>any</u> fragment of the single chain polypeptide, but <u>only</u> embraces those fragments that have the requisite native clostridial neurotoxin light chain activity, namely the ability to proteolytically cleave one or more vesicle or plasma-membrane associated proteins essential to exocytosis. The latter activity may be readily confirmed by any one of a number of routine assays (see below).

Claim 1 further qualifies the single chain polypeptide fragment as having the native activity of clostridial neurotoxin heavy chain H_N. Thus, Claim 1 does not embrace <u>any</u> fragment of the single chain polypeptide, but <u>only</u> embraces those fragments having the requisite clostridial neurotoxin heavy chain H_N activity, namely the ability to translocate the polypeptide into a cell and/ or to increase the solubility of the polypeptide as compared with the solubility of the first domain alone. These activities may be readily confirmed by any one of a number of routine assays (see below).

These results confirm that the amino acid sequence of the native clostridial neurotoxin L-chain polypeptide sequence (first domain) may be modified (eg. by introducing substitutions, additions or deletions) whilst retaining the protease activity of the native clostridial neurotoxin L-chain.

➢ Clostridial neurotoxin Heavy chain H_N activity

It is routine to confirm that a clostridial neurotoxin H_N polypeptide (or a fragment or variant thereof as defined in the pending claims) has the required translocation function.

In this regard, I refer to the attached Annex 2 (and accompanying publications by Shone *et al.* and Blaustein *et al.*), which discusses suitable conventional assays for detecting clostridial neurotoxin H_N polypeptide translocation activity. In use of these assays, the presence of active translocation function is confirmed by monitoring the flow of ions across a liposome or phospholipid bilayer membrane.

With respect to the ability of a clostridial neurotoxin H_N polypeptide (or fragment or variant) to increase the solubility of a L-chain (or equivalent L-chain component), I refer to the simple, conventional assays described on page 8 of the specification as filed.

As confirmed by Example 2 of the specification as filed, a botulinum H_N polypeptide fragment having only 107 residues was sufficient to maintain solubility of a single-chain polypeptide (LH_{107}/B, SEQ ID NO: 24).

Thus, it would be a matter of routine to identify which residues of clostridial neurotoxin heavy chain H_N polypeptide may be deleted or modified, whilst retaining the desired translocation and/ or solubilising activity.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false

statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that willful, false statements may jeopardize the validity of the application or any patent issued thereon.

Signed ..... (C. STOWE)

Witnessed ..... (CARLS E. NWOGUH)

Dated ..... 23/11/09 .....

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. DO NOT EXCEED FOUR PAGES.

NAME	POSITION TITLE
Clifford Charles Shone	**Scientific and Program Leader**
eRA COMMONS USER NAME cliffcshone	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Liverpool, Liverpool, UK	B.Sc. (hon)	1975	Biochemistry
University of Liverpool, Liverpool, UK	Ph.D.	1978	Biochemistry

A. Positions and Honors

Previous Employment

1978-1981	Postdoctoral Fellow at Dept of Biochemistry & Biophysics, Iowa State University, Ames, Iowa, USA
1981-1987	Senior Scientist at Centre for Applied Microbiology & Research (CAMR), Porton Down, Salisbury, Wiltshire, UK
1987-1997	Head of Section (Protein Toxins) at CAMR
1997-present	Scientific and Programme Leader at Health Protection Agency (HPA, formerly CAMR), Porton Down, Salisbury, Wilts. SP4 0JG, UK

Summary of relevant skills and experience

Managerial:- Project management and leading scientific teams; staff recruitment; preparing grant applications, papers and patents.

Technical:- Protein (toxins) and peptide chemistry (purification, analysis, labelling, conjugation); enzymology and enzyme kinetics; ligand-receptor analysis; immunoassays; basic molecular biology; basic cell culture

B. Selected Peer-reviewed Publications

(selected from 60 peer-reviewed publications)

1. Schiavo, G., Shone, C.C., Rossetto, O., Alexander, F.C.G., and Montecucco, C. (1993) Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin, *J. Biol. Chem.* 268, 11516-11519.
2. Shone, C.C., Quinn, C.P., Wait, R., Hallis, B., Fooks, S.G., Hambleton, P. (1993) Proteolytic cleavage of synthetic fragments of vesicle-associated membrane protein, isoform-2 by botulinum type B neurotoxin, *Eur. J. Biochem,* 217, 965-971.
3. Shone, C.C and Roberts, A. (1994) Peptide substrate specificity and properties of the zinc endopeptidase activity of botulinum type B neurotoxin *Eur. J. Biochem* 225, 263-270.
4. Cenci di Bello, I., Poulain, B., Shone, C.C., Tauc, L., and Dolly, J.O. (1994) Antagonism of the intracellular action of botulinum type A neurotoxin with mAbs that map to light chain epitopes. *Eur. J.Biochem.* 219, 161-169.
5. Rosetto, O., Schiavo, G., Montecucco, C., Poulain, P., Deloye, F., Lozzi, L. and Shone, C.C. (1994) SNARE motifs and neurotoxins, *Nature,* 372, 415-416.
6. Foran, P., Shone, C.C. and Dolly, J.O. (1994) Differences in the proteolytic activity of tetanus and botulinum type B toxins revealed by cleavage of VAMP and various sized peptides, *Biochemistry,* 33, 15365-15374.

7. Schiavo, G., Shone, C.C., Bennett, M., Scheller, R., and Montecucco, M., (1995) Botulinum neurotoxin type C cleaves at single Lys-Ala bond within the carboxyl-terminal region of syntaxin, *J. Biol. Chem.* 270, 10566-10570.
8. Boyd, R.S., Duggan, M.J., Shone, C.C., and Foster, K.A. (1995) The effect of botulinum neurotoxins on the release of insulin from insulinoma cell lines HIT-15 and RINm5F *J. Biol. Chem. (commun.)* 270, 18216-18218
9. Foran, P., Lawrence, G.W., Shone, C.C., Foster, K., and Dolly, J.O. (1995) BoNT/C1 cleaves both syntaxin and SNAP-25 in intact and permeabilised cells. *Biochemistry*, 35, 2630-2636.
10. Wictome, M., Rossetto, O., Montecucco,C., and Shone , C.C. (1996) Substrate residues N-terminal to the cleavage site of botulinum type B neurotoxin play a role in determining the specificity of its endopeptidase activity. *FEBS Lett.*, 386, 133-136.
11. Hallis, H., James B.A.F., and Shone, C.C. (1996) Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities. *J. Clin. Microbiol.* 34, 1934-1938.
12. Pellizzari, R., Rossetto, O., Lozzi, L., Giovedi, S., Johnson, E., Shone, C.C., and Montecucco, C. (1996) Structural determinants of the specificity for synapatic vesicle-associated membrane protein/synaptobrevin of tetanus and botulinum type B and G neurotoxins. *J. Biol.Chem.* 271, 20,353-20,358.
13. Pellizari, R., Maason, S., Shone, C.C., and Montecucco, C. (1997) The interaction of synaptic VAMP/synaptobrevin with botulinum neurotoxins D and F. *FEBS Lett.* 409, 339-342.
14. Chen, F., Foran, P., Shone , C.C., Foster, K., Melling, J. and Dolly O. (1997) Botulinum type B inhibits insulin-stimulated glucose uptake into adipocytes and cleaves cellubrevin unlike type A toxin which failed to proteolyze the SNAP-23 present. *Biochemistry*, 36, 5719-5728.
15. Wictome, M., Newton, K.A., Jameson, K., Dunnigan, P., Clarke, S. Gaze, J., Tauk, A., Foster, K.A. and Shone, C.C. (1999) Development of *in vitro* assays for the detection of botulinum toxin in foods. *FEMS Immunol. Med. Microbiol.*, 24, 319-323.
16. Wictome, M., Newton, K.A., Jameson, K., Mackay, E., Clarke, S., Taylor, R., Gaze, J., Foster, K., and Shone, C.C. (1999) Development of an *in vitro* assay for botulinum type B toxin that is more sensitive than the mouse bioassay. *Appl. Environ. Microbiol.* 65, 3787-3792.
17. Chaddock, K., Purkiss, J., Friss, L., Broadbridge, J., Duggan, M., Shone, C., Quinn, C., and Foster, K. (2000) Inhibition of neurotoxin release by a retargeted endopeptidase derivative of *C. botulinum* type A. *Infect Immun.*, 68, 2587-2593.
18. Chaddock, J.A., Purkiss, J.R., Duggan, M.J., Quinn, C.P., Shone, C.C. and Foster, K.A. (2000) A conjugate composed of nerve growth factor and a non-toxic derivative of botulinum neurotoxin type A can inhibit neurotransmitter release *in vitro*. *Growth Factors*, 18, 147-155.
19. Roberts, A.K. and Shone, C.C. (2001) Modification of surface histidine residues abolishes the cytotoxic activity of *Clostridium difficile* toxin A. *Toxicon* 39, 325-333.
20. Sutton, J.M., Chow-Worn, O., Spaven, L., Silman, N.J., Hallis, B. and Shone, C.C. (2001) Tyrosine-1290 of tetanus neurotoxin plays a key role in its binding to gangliosides and functional binding to neurons. *FEBS Lett.*, 493, 45-49.
21. Chaddock, J.A., Herbert, M.H., Ling, R.J., Alexander, F.C., Fooks S.J., Revell, D.F., Quinn, C.P., Shone, C.C., Foster, K.A. (2002) Expression and purification of catalytically active, non-toxic endopeptidase derivatives of *Clostridium botulinum* toxin type A. *Protein Expr Purif.*, 25, 219-228.
22. Duggan, M.J., Quinn C.P., Chaddock, J., Purkiss, J.R. , Alexander, F., Doward, S., Fooks, S.J., Friis, L., Hall, Y., Kirby, E., Leeds, N., Moulsdale, H.J. Dickenson, A., Green, M., Rahman, W., Suzuki, R., Shone C.C., and Foster, K.A. (2002) Inhibition of release of neurotransmitters from rat dorsal root ganglia by a novel conjugate of a *Clostridium botulinum* toxin A endopeptidase fragment and *Erythrina cristagalli* lectin. *J. Biol. Chem. 277, 34846-34852.*
23. Stancombe, PR, Alexander, FCG, Ling, RJ, Matheson, MA, Shone CC & Chaddock, JA. (2003) Isolation of the gene and large-scale expression and purification of recombinant Erythrina cristagalli lectin. Protein Expression & Purification, 30, 283-292.
24. Evans HR, Sutton JM, Holloway DE, Ayriss J, Shone CC, and Acharya KR. (2003) The crystal structure of C3stau2 from Staphylococcus aureus and its complex with NAD. *J Biol Chem.* 278:45924-45930.

25. Sutton, JM, Wayne, J, Scott-Tucker, A, O'Brien, SM, Marks, PMH, Alexander, FCG, Shone, CC and Chaddock, JA (2005) Preparation of specifically activatable endopeptidase derivatives of Clostridium botulinum toxins type A, B and C and their applications. Protein Expression & Purification 40: 31-41.
26. Holbourn, KP, Sutton, JM, Evans, HR, Shone, CC, Acharya, KR (2005) Molecular recognition of an ADP-ribosylating Clostridium botulinum C3 exoenzyme by RalA GTPase. Proc Natl Acad Sci U S A. 102:5357-5362.
27. Evans, ER, Sutton, JM, Gravett A, Shone, CC. (2005) Analysis of the substrate recognition domain determinants of Botulinum Type B toxin using Phage Display. Toxicon, 15: 446-53
28. Foster KA, Adams EJ, Durose L, Cruttwell CJ, Marks E, Shone CC, Chaddock JA, Cox CL, Heaton C, Sutton JM, Wayne J, Alexander FC, Rogers DF. (2006) Re-engineering the target specificity of Clostridial neurotoxins - a route to novel therapeutics. Neurotox Res;9:101-107.

29. Shone C, Ferreira J, Boyer A, Cirino N, Egan C, Evans E, Kools J, Sharma S. (2006)) The 5th International Conference on Basic and Therapeutic Aspects of Botulinum and Tetanus Neurotoxins. Workshop review: assays and detection. Neurotox Res. 9: 205-216.

C. RESEARCH SUPPORT

Ongoing Research (grant & contract support)

Title and Reference: LH_N Recombinant Botulinum Vaccine program
Funding body: Emergent BioSolutions.; 2003- present
Role: PI

Completed Research (grant support)

Title and Reference: Sensitive assays for botulinum neurotoxins
Funding body: NIAID/NIH (R01 AI055578); 09/01/03-12/31/05
Role: PI

Title and Reference: Motor neuron vectors (Shone/Apr99/067)
Funding body: Motor Neurone Disease Association; 6/12/99-6/11/00
Role: PI

Title and Reference: Development of peptide inhibitors of the botulinum neurotoxins (CU013-0000004558)
Funding body: Ministry of Defence; 11/01/99-1/01/03
Role: PI

Title and Reference: Development of neuronal-specific targeting ligands
Funding body: Wellcome Trust; 10/01/01-9/30/03
Role: PI

Annex 1

Confirmation of L-chain function by assaying for protease activity inherent to the L-chain.

A skilled person would be aware of a number of routine methods for confirming that a particular clostridial neurotoxin L-chain (or fragment or variant thereof) has the requisite protease activity. Thus, prior to the present invention, it would have been routine for a skilled person to determine the presence of a "first domain" falling within the scope of the pending claims.

For example, in a rough-and-ready test, SNAP-25 (or synaptobrevin, or syntaxin), which are natural substrates of the protease activity of the clostridial neurotoxin L-chain, may be challenged with a test fragment or variant polypeptide, and then analysed by SDS-PAGE peptide separation techniques. Subsequent detection of peptides (eg. by silver staining) having molecular weights corresponding to the cleaved products of SNAP-25 (or other component of the neurosecretory machinery) would indicate the presence of an L-chain (or a fragment or variant thereof) possessing the requisite protease activity.

Alternatively, protease activity may be monitored by disappearance of the L-chain substrate.

As a further alternative, L-chain function may be assayed for by detecting protease cleavage products using of the antibodies that selectively bind to a product of the peptide cleavage reaction. Such antibodies are described in, for example, PCT/GB95/01279 (ie. WO95/33850) in the name of the present Applicant. In more detail, a specific antibody is employed for detecting cleavage of SNAP-25. Since the antibody recognises cleaved SNAP-25, but not uncleaved SNAP-25, identification of the cleaved product by the antibody confirms the presence of an L-chain (or a fragment thereof) possessing the requisite proteolytic activity as recited in the pending claims.

Annex 2

Confirming H_N function by assaying for translocation activity inherent to H_N.

A skilled person would be aware of a number of routine methods for confirming that any fragment or variant polypeptide has the required H_N activity. Suitable methods are, for example, described by Shone *et al.* (1987) Eur. J. Biochem. 167, pp.175-180, and by Blaustein *et al.* (1987) FEBS 226 (1), pp.115-120 (enclosed).

The Shone *et al.* method employs artificial liposomes loaded with potassium phosphate buffer (pH 7.2) and radiolabelled NAD. Release of K^+ and NAD from the liposomes correlates with a positive result for channel forming activity and hence translocation activity. In this regard, K^+ release from liposomes may be measured using an electrode and NAD release calculated by measuring the radioactivity in the supernatant (see page 176, column 1, line 33 - column 2, line 17).

The Blaustein *et al.* method employs planar phospholipid bilayer membranes, which are used to test for channel forming activity. In more detail, salt solutions on either side of the membrane are buffered at a different pH - on the *cis* side, pH 4.7 or 5.5 and on the *trans* side, pH 7.4. The "agent" to be tested is added to the *cis* side of the membrane and electrical measurements are made under voltage clamp conditions, in order to monitor the flow of current across the membrane (see paragraph 2.2, pages 116-118). The presence of an active translocation function is confirmed by a steady rate of channel turn-on (i.e. a positive result for channel formation) - see paragraph 3, page 118.